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BIOCHEMICAL BASIS OF VIRULENCE IN EPIDEMIC TYPHUS

Annual and Final Report

Herbert H. Winkler, Ph.D.

January 1983

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University of South Alabama

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20. ABSTRACT

ABSTRACT

The purpose of the project is to elucidate the biochemical basis of virulence in epidemic typhus. The basic method is a comparison of the E strain and Breinl strain (avirulent and virulent, respectively) of Rickettsia prowazekii in their interaction with the host defense system.

This is a final report on activities commencing January 1, 1979 to 30 April 1981. Annual reports submitted in August 1979 and 1980 are on file and detail the activities of those years. This report will only summarize those projects.

Subsequent to the last annual report our work has focused on two areas 1) a study of the ability of virulent and avirulent Rickettsia prowazekii in the **cytoplasm** of macrophage-like cell lines which employed a fusion technology and 2) the preparation of a manuscript on the macrophage-like cell lines' ability to differentiate the virulent and avirulent typhus rickettsiae. This latter work has now been published in the journal, Infection and Immunity, and the book, Rickettsiae and Rickettsial Diseases. A note describing the fusion work has been submitted for publication.

In previous years the avirulent, Madrid E, and virulent, Breinl, strains of Rickettsia prowazekii were compared with respect to their interaction with a) mouse macrophage-like cell lines, b) a human macrophage-like cell line, c) mouse peritoneal macrophages and d) guinea pig peritoneal macrophages. Furthermore, the fatty acid composition and plasmid DNA content of these strains were compared and the interaction of R. prowazekii with rabbit polymorphonuclear leukocytes was also detailed and the latter published in Infection and Immunity.

SUMMARY

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The mouse macrophage-like cell lines and the guinea pig peritoneal macrophages could differentiate between the strains. The avirulent strain was eliminated and the virulent strain grew within the cytoplasm of these cells. The human macrophage-like cell line and the mouse peritoneal macrophages, on the

other hand, could not distinguish between the two strains. Mouse macrophages destroyed both strain and both strains grew in the human macrophage-like cell line. The mechanism that the macrophages use to differentiate between the virulent and avirulent strains had become a key goal when the project was terminated.

The fatty acid composition of the two strains were compared. The hypothesis was that the high level of unsaturated fatty acid known to be in the avirulent strain might not occur in the virulent strain and that this might be a target for selective killing via lipid peroxidation. Our investigation established that fatty acid composition was not different in the two strains.

Plasmids are often a source of virulence factors. The two strains were compared and neither was found to have detectable plasmid DNA.

Infected L-cells which are permissive for the growth of both virulent and avirulent R. prowazeki were fused with RAW-264 macrophage-like cells which allow the growth of only virulent rickettsiae killing the avirulent rickettsiae which infect them. The formation of a fused L-RAW heterokaryon allows the rickettsiae to enter the macrophage without phagocytosis. These experiments show that the avirulent rickettsiae are killed by the cytoplasm of the heterokaryons to a much greater extent than the virulent rickettsia. Thus, the selective survival of the virulent strain in macrophage involves interaction with host at the cytoplasmic level after escape from the phagosome.

FOREWARD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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PROGRESS REPORT

I. Interactions Between Rickettsia prowazekii and Rabbit Polymorphonuclear Leukocytes: Rickettsiacidal and Leukotoxic Activities

by: T. Stuart Walker and Herbert H. Winkler

These studies are published in: Infection and Immunity, 31:289(1981)

Rickettsia prowazekii was assessed for in vitro susceptibility to phagocytosis by rabbit polymorphonuclear leukocytes. (a-^{32}P)adenosine triphosphate-labeled rickettsiae were used to determine phagocytosis and adsorption quantitatively. *R. prowazekii* was less susceptible to phagocytosis than were *Escherichia coli* and *Neisseria gonorrhoeae*. Although *R. prowazekii* was similar to *E. coli* in susceptibility to superoxide and activated halide, few phagocytized rickettsial cells were inactivated after being ingested by polymorphonuclear leukocytes, and rickettsiae were observed free in polymorphonuclear leukocyte cytoplasm. At low ratios of rickettsiae to polymorphonuclear leukocytes, PMN phagocytosis increased as a linear function of time, but at high ratios (multiplicity of infection=50) rickettsiae were phagocytized during only the first 10 min of incubation. Polymorphonuclear leukocytes were damaged in the presence of high rickettsial multiplicities such that they released lactate dehydrogenase into the medium and lost the ability to phagocytize both rickettsiae and *E. coli*. The amount of leukotoxic activity in a given rickettsial sample correlated with the relative hemolytic activity of that sample. The rickettsial leukotoxin was probably not a soluble product, was active in the absence of phagocytosis, and was inhibited by inactivation of the rickettsiae or by incubation at 4°C.

II. Absence Of Detectable Plasmid DNA In Madrid E And Breinl Strains Of Rickettsia Prowazekii

by David O. Wood and Herbert H. Winkler

Plasmids are circular pieces of DNA that exist extrachromosomally in bacterial cells. Although they are generally considered nonessential to the survival of the host cell, they confer a variety of phenotypic properties that can impart advantages to the bacterium. Such properties include antibiotic resistance, toxin production, fertility, bacteriocin production, and production of virulence factors. (4). Numerous examples of plasmids conferring such phenotypes are found both in gram-positive and gram-negative bacteria.

Plasmids exist within the bacterial cell in the form of a covalently closed circular (CCC) molecule of DNA which replicates independently of the chromosome (1). This physical configuration has been exploited for the purpose of isolating plasmid DNA by cesium chloride-ethidium bromide density gradients (3). Plasmid DNA in the form of CCC molecules binds less ethidium bromide than chromosomal DNA and bands at a denser position. The DNA bands can be visualized with ultraviolet light and the plasmid band removed. After extraction of the ethidium bromide, this procedure results in a pure preparation of plasmid DNA. Generally, however, initial detection of plasmids is achieved by subjecting cell lysates to agarose gel electrophoresis (2). Purified plasmid DNA from CsCl-EB gradients can also be characterized by this method. DNA subjected to agarose gel electrophoresis migrates according to size and configuration and can be visualized by staining the gel with ethidium bromide.

Rickettsia prowazekii is an obligate intracellular bacterium which is the causative agent of epidemic typhus in man. This bacerium's unusual intracellular existence, the mechanism by which it enters eucaryotic cells, and

its virulence properties have prompted numerous investigations. Since plasmid DNA is associated with surface antigens and virulence factors, we turned our attention to an examination of plasmid DNA in two well-characterized strains of R. prowazekii, the avirulent Madrid E strain and the virulent Breinl strain.

The virulent Breinl and avirulent Madrid E strains of Rickettsia prowazekii were examined for the presence of plasmid DNA by cesium chloride-ethidium bromide density equilibrium centrifugation and by agarose gel electrophoresis of small volume, crude lysates. No plasmid DNA was detected in either strain using these techniques. Thus although it could be expected that less-passaged isolates might be more likely to have plasmids, there is no correlation with virulence in the E-Breinl system.

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III. Selective Elimination of the Avirulent, Madrid E, strain of *Rickettsia prowazekii* by Guinea Pig Peritoneal Macrophages.

by Herbert H. Winkler and Robin M. Daugherty

The guinea pig has been the most widely used animal model for the study of the pathogenesis of epidemic typhus. Only the cotton rat, which is not readily available as a laboratory strain in this country, is an alternative. Mice and albino rats have been shown to be uninfectible by *Rickettsia prowazekii* and we have extended this list to include the golden hamster and gerbil.

An avirulent strain, the Madrid E. strain, of *R. prowazekii* can be distinguished from the common virulent (Breinl) strain by its ability to immunize guinea pigs and man without causing mortality of significant morbidity. However, the biochemical basis of virulence in *R. prowazekii* is unknown. To date the only published *in vitro* data bearing on this are those of Gambrill and Wisseman who showed that cultured human monocytes can destroy the avirulent strain but that the virulent strain grows within the phagocytes. However, we have in preparation studies of rickettsial interaction with mice and macrophage-like cell lines.

We have investigated the peritoneal macrophage of guinea pigs to develop a flexible animal model for the study of pathogenesis and the basis of virulence. The oyster shell glycogren elicited peritoneal macrophage of the guinea pig interacts differently with the avirulent E strain and the virulent Breinl strain of *Rickettsia prowazewkii*. 24 h after a wide range of initial infections the

Breinl strain survives in about 71% of those macrophages it infected and the number of rickettsia per macrophage increases about four-fold. The E strain, on the other hand, survives in only about 9% of those macrophages it infected and the number of rickettsiae per macrophage falls to about 13% of that present initially.

IV. Comparison of the Fatty Acid Composition of Avirulent and Virulent Strains of *Rickettsia prowazekii*

by Herbert H. Winkler and Elizabeth T. Miller

The rickettsiae were harvested from infected chicken egg yolk sac, purified through Renografin, the lipids were extracted with chloroform-methanol, the fatty acids of the phospholipids were cleaved and converted to methyl esters with methanolic HCl and the methyl esters were separated and quantitated by gas liquid chromatography, all as previously described by Winkler and Miller, (J. Bacteriol. 136:175, 1978).

The phospholipids of the avirulent and virulent strains of *R. prowazekii* contain the same fatty acids. The slightly higher levels of 16:0 and 18:0 in the Breinl strain are probably not significant since variation in the phosphatidyl choline content of the rickettsial preparation (which, in the E strain, we showed was host derived) would be most strongly reflected in these fatty acids. The fatty acid composition of the individual phospholipid classes (PE, PG, PC) was not determined for the Breinl strain. However, published data for the E strain showed little differences in the fatty acids of PE and PG, the major phospholipids of this strain (Winkler and Miller).

R. prowazekii, Madrid E strain, was shown to have a surprisingly high percentage of its fatty acids unsaturated (Winkler and Miller). We hypothesized that the E strain was much higher in percentage of unsaturated fatty acids than the Breinl strain. This could account for a greater susceptibility to destruction through lipid peroxidation in the E strain. The E strain is selectively eliminated relative to the Breinl strain in human monocytes, (Gambrill and Wisseman) guinea pig macrophages (Winkler and Daugherty) and mouse macrophage-like cell lines (Turco and Winkler). The results do not support this hypothesis.

V. Clearance of *Rickettsia prowazekii* by Mouse Peritoneal Macrophages

by Herbert H. Winkler and Robin Daugherty

A role for the macrophage in the pathogenesis of rickettsial diseases is strongly suggested. Nacy et al. (5,6) showed that activated mouse macrophages *in vitro* could eliminate *R. tsutsugamushi* but that resident macrophages could not. Gambrill and Wisseman (4) showed that cultured human monocytes could kill the avirulent strain of *R. prowazekii*. However, the virulent strains of *R. prowazekii* and *R. typhi* grew within these monocytes unless these virulent strains had been reacted with antiserum in which case they were destroyed by the monocyte (1,3,4).

The advantages of the mouse peritoneal macrophage as a model for the pathogenesis of epidemic typhus infections and as a means of exploring the differences in the virulent and avirulent strains of *R. prowazekii* are numerous. Little has been described on the interaction of mouse macrophages and *R. prowazekii*.

In this study we describe the interactions of the E strain and Breinl strain of *R. prowazekii* with resident, elicited and activated peritoneal macrophages from three strains of mice including the "beige" strain which has impaired lysosomal degranulation (2).

Mouse peritoneal macrophages can clear their cytoplasm of *R. prowazekii* in 24 to 48 hours. This clearance is rapid in onset: by 4 hrs after infection a significantly lower fraction of the macrophages have any rickettsia within them. Any macrophages, unable to clear the rickettsiae by 24 to 48 hrs, a number in general less than 5%, are no longer even rickettsiastatic and the number of rickettsiae within these few macrophages increases. These rickettsiae will eventually fill and kill these macrophages.

The macrophages of both BALB/c and C57/6B1 mice were able to kill intracellular rickettsiae. Activation of the macrophages was unnecessary; resident and oyster shell glycogen elicited macrophages were as rickettsiacidal as those activated by the administration of C. parvum or immunization with R. prowazekii of either strain. Alterations in activation-state as a result of in vitro cultivation of the macrophages was also not crucial since macrophages which had been cultivated for seven days, 24 hrs or infected with rickettsiae the same day they were removed from the mouse were all able to kill rickettsiae.

Both the virulent Breinl strain and the avirulent E strain were eliminated from the macrophages and no tendency for survival or growth of the virulent strain relative to the avirulent strain was present in any of the macrophage types. Opsonization of either the E or Breinl strains resulted in more phagocytosis and hence a greater initial infection but no effect on the subsequent clearance of these rickettsiae was evident.

The beige mutant of C57/6 has a defect in degranulation and its neutrophils are unable to kill bacteria as well as those of the wild type mouse. The oyster glycogen elicited peritoneal macrophages of these mice were found to contain giant lysosomes when stained by acridine orange as has been seen in their neutrophils by other groups. In spite of this defect, the clearance of both avirulent and virulent rickettsiae occurred, and in this series of experiments was slightly better than in the macrophages of the wild type mouse.

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VI. Differentiation between Virulent and Avirulent Strains of *Rickettsia prowazekii* by Macrophage-like Cell Lines

by Jenifer Turco and Herbert H. Winkler

These studies are published in:

1. **J. Turco and H.H. Winkler. Interaction of *Rickettsia prowazekii* and macrophage-like cell lines. *In Rickettsiae and Rickettsial Diseases.* (Burgdorfer, W. and Anacker, R., eds.). p. 81 ff. Academic Press.**
2. **J. Turco, and H.H. Winkler. Differentiation between virulent and virulent strains of *Rickettsia prowazekii* by macrophage-like cell lines. Infect. Immun. 35:783 (1982).**

Ability to resist destruction in the phagocytic cells of the host is a definite advantage for an obligate intracellular parasite. Yet relatively few studies have dealt with the fate of *Rickettsia* species in macrophages.

Nacy and Osterman (7) recently reported that *Rickettsia tsutsugamushi* multiplied within resident mouse peritoneal macrophages, and that treatment of the rickettsiae with immune serum prior to infection of the macrophages led to destruction of many (though not all) of the rickettsiae within the macrophages. In addition, they along with Nacy and Meltzer (6), provided evidence that activated macrophages are involved in host defense against *R. tsutsugamushi*.

Gambrill and Wisseman (4) showed that both *Rickettsia typhi* and the virulent Breinl strain of *Rickettsia prowazekii* could multiply in human monocyte-derived macrophages. Incubation of either organism with immune serum prior to infection of human macrophages resulted in destruction of the rickettsiae within the cells (1,3). Unlike the virulent Breinl strain, the avirulent E strain of *R. prowazekii* failed to multiply in most human macrophages (4). This observation suggested that detailed study of the interaction of *R. prowazekii* and macrophages might allow definition of the basis for the

difference in virulence between the E and Breinl strains.

In recent years several continuous macrophage-like cell lines have been developed (2,5,8). Each cell line exhibits some, though not all, of the properties characteristic of macrophages. Because these cells can easily be grown in culture and would readily lend themselves to a variety of studies, we have examined the interaction of avirulent And virulent strains of R. prowazekii with several macrophage-like cell lines.

The growth of avirulent (E) and virulent (Breinl) strains of Rickettsia prowazekii was compared in four mouse macrophage-like cell lines (RAW264.7, J774.1, P388D1, and PU5), one human macrophage-like cell line (U937-1), and the mouse fibroblast line L929. The E and Breinl strains grew equally well in L929 cells. However, all of the mouse macrophage-like cell lines clearly differentiated between the two strains by restricting the growth of the E strain relative to that of the Breinl strain. A nonuniform response to infection was sometimes observed in which E strain rickettsiae were cleared from the majority of the infected cells, but multiplied in some of the remaining infected cells. The human line U937-1 was not very effective at differentiating the E and Breinl strains. Addition of rabbit antirickettsial antiserum to the Breinl or E strains of R. prowazekii immediately before infection of L929 cells caused a marked decrease in the initial infection but had no effect on the subsequent growth of the rickettsiae in the L929 cells. In contrast, addition of antiserum to Breinl or E strain rickettsiae immediately before infection of macrophage-like cell lines caused either no change or an increase in the initial infection. Most of the rickettsiae that infected the mouse macrophage-like cell lines in the presence of antiserum were destroyed in these cell lines. Thus, when the infection took place in the presence of antiserum, the mouse macrophage-like cell lines no longer differentiated between the E and Breinl strains. These

data indicate that mouse macrophage-like cell lines should be a useful model system for defining the differences between the E and Breinl strains of Rickettsia prowazekii, differences which should lead to an understanding of the biochemical basis of virulence in this organism.

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VII. Cytoplasmic Distinction of Avirulent and Virulent Rickettsia prowazekii: Fusion of Infected Fibroblasts (L-929 Cells) with Macrophage-like Cells (RAW264.7).

by: Herbert H. Winkler and Robin M. Daugherty

L-929 cells with either virulent or avirulent Rickettsia prowazekii growing in their cytoplasm were fused with cells of the macrophage-like cell line, RAW 264.7. Fusion occurred between the two cell types producing heterokaryons containing R. prowazekii. Both the number of rickettsiae per infected heterokaryon and the percentage of heterokaryons infected with rickettsiae decreased in experiments with the avirulent strain relative to the virulent strain. These results indicate that the differential survival of the virulent strain in macrophages is some yet to be defined function of the cytoplasm of the macrophage and that the distinction is not just at the phagolysosome level.

Rickettsia prowazekii are obligate intracellular bacteria that can grow only within the cytoplasm of their eukaryotic host cell. Unlike facultative intracellular bacteria, rickettsiae can escape from the phagosomes. Rickettsiae never grow bounded by a phago(lyso)somal membrane. Although both the avirulent and virulent strains of R. prowazekii replicate equally well in the cytoplasm of non-professional phagocytes, only the virulent strain thrives in human macrophages and murine macrophage-like cell lines (1,4). The avirulent strain

not only fails to replicate but is cleared from such cells.

In this study we have examined whether the cytoplasm of the murine macrophage-like cell line RAW264.7 can distinguish the avirulent (E) from the virulent (Breinl) strain of *R. prowazekii*. The alternative is that all distinction is made in the phago(lyso)somal vesicle during the rickettsia's transient passage from the medium to the cytoplasm. To answer this question it was necessary to introduce the rickettsial strains into the cytoplasm of the macrophage without the formation of a phago(lyso)somal vesicle since the interaction of the two strains with the milieu of these vesicles might have affected their subsequent fate within the cytoplasm. Therefore, the rickettsial strains were grown in L-929 cells (a cell permissive for both strains) which were then fused with RAW264.7 (a cell permissive for only the virulent strain), and the growth of the rickettsial strains in these heterokaryons was compared.

The rickettsiae were grown in chick embryo yolk sacs and purified as previously described (4,5). L-929 cells were grown and were irradiated to prevent replication of the host cells as previously described (4), and were then infected in suspension with the indicated strain of rickettsiae at a multiplicity of about 40 bacteria per eukaryotic cell. The L-929 cells were washed thrice, then resuspended in Dulbecco modified Eagle medium (DMEM) with 10% calf serum. Small samples were cytofuged to determine the initial infection parameters. 1 ml portions ($2x10^5$ cells) were planted in 35 mm dishes containing three 13 mm cover slips. This concentration resulted in well-separated cells on the cover slips. After 18 h incubation, $6x10^5$ irradiated but uninfected L-929 or RAW264.7 cells were added to the dishes containing the infected cells on cover slips and incubated at 34°C for 3 h. Nonadherent cells were removed by gentle washing, and the coverslips held in sterile forceps were each dipped in a 10% polyethyleneglycol (mol. wgt., 4000;PEG) solution in DMEM for 60 s, washed, and placed in a 35 mm dish with DMEM for another 24 h. After this time the

cover slips were removed and stained by a modification of the Gimenez method (3,6).

In preliminary experiments, the infected L-929 cells on the cover slips were stained with Hoescht #33342 dye and the noninfected cells added were stained with nuclear yellow. These experiments showed no fused cells containing multiple Hoescht-stained nuclei, but about 24% of the cells were heterokaryons with Hoescht- and nuclear yellow-stained nuclei. A higher efficiency of fusion could be obtained using higher concentrations of PEG but these concentrations were toxic for the rickettsiae.

The Gimenez-stained cover slips from appropriate experiments were scored for a) percentage of heterokaryons infected with rickettsiae, b) number of rickettsiae per infected heterokaryon, c) number of rickettsiae per residual (unfused) single L-929 cells in the fusion experiments, d) percentage of control (no PEG) L-929 cells infected with rickettsiae and e) the number of rickettsiae per infected control (no PEG) L-929 cell. At least 200 cells or heterokaryons were counted to determine these values. The averages in the tables were based on eight experiments.

Table I shows the effect of fusion on the percentage of heterokaryons infected with each of the two strains of rickettsiae. In the unfused L-929 cells about 60% of the cells were infected with each strain of rickettsiae at 24 h post-fusion and 45 h post-infection. This was the same percentage as at fusion. When uninfected L-929 cells were fused with the infected L-929 cells, 67% of the infected cells remained positive for rickettsiae of either strain. Although it did not show any strain specificity, this fall to 67% was still unexpected. However, when uninfected RAW264.7 cells were fused with the infected L-929 cells a marked strain difference was observed in these heterokaryons. The percentage of heterokaryons infected remained high (72% of

the unfused control) when infected with the virulent Breinl strain which can grow within the macrophage but this percentage fell to 44% of the unfused control in experiments with the E strain which cannot survive in these macrophages.

In Table 2 the ability of these two strains of rickettsiae to grow in these heterokaryons was examined. The unfused L-929 cells and the residual single cells all had about 40 rickettsiae per infected cell. These values were independent of the rickettsial strain used and represented about a five-fold increase during the preceding 24 h. Similarily, both the Breinl and E strains grew slightly less well (87% of control) in the L-929:L-929 fused cells. In contrast, a marked strain difference was seen following fusion with the macrophage-like cell line. Less than half as many avirulent rickettsiae as virulent rickettsiae were found in the L-929:RAW264.7 heterokaryons. The specific loss in avirulent rickettsiae can be seen in this column of the table. Surprisingly, even the Breinl strain which can grow well in both L-929 and RAW264.7 cell increased in numbers less well (60%) in the heterokaryon formed from the fusion of these two cell types.

These results show that the virulent Breinl strain both survives and replicates better when introduced into the cytoplasm of L-929:RAW264.7 heterokaryons than does the avirulent E strain. Thus, the mechanism for the selective survival and growth of the virulent organism must extend beyond interactions at the phago(lyso)somal membrane level. Both the Breinl and E strains are able to lyse eukaryotic cell membranes via a phospholipase A and thus can probably escape from the vesicle to the cytoplasm (5). Thus, the phagolysosome may have a role only in destroying opsinized rickettsiae (2).

The existence of bactericidal factors has not been described in macrophage cytoplasm since bacteria, except rickettsiae, are confined to extracellular or intravesicular existence. The failure of the avirulent strain to thrive in

macrophage cytoplasm may represent either the effect of a rickettsicidal factor in the macrophage cytoplasm to which the virulent strain is resistent or a basic biochemical lesion in the avirulent strain which prevents its growth in the cytoplasm of the macrophage but not the L-929 cell.

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Table 1: Effects of fusion on percentage of cells infected with *R. prowazekii*

Cells	Rickettsial Strains: Breinl (avirulent)	Rickettsial Strains: E (avirulent)	Specific Loss[c]
Unfused L-929	63 ± 5[a] (100)[b]	58 ± 5 (100)	-
L-L Heterokaryons	40 ± 5 (67 ± 8)	38 ± 5 (67 ± 8)	1.0
L-R Heterokaryons	43 ± 3 (72 ± 7)	25 ± 4 (44 ± 6)	2.0

a) Mean and standard error. Parameters were determined 24 h post-fusion, 45 h post-infection.

b) The numbers in parentheses are normalized to the unfused L-929 cells in each experiment.

c) Specific Loss = % loss with E strain ÷ % loss with Breinl strain.

Table 2: Effects of fusion on the number of *R. prowazekii* per infected cell.

Cells	Rickettsial Strains: Breinl (virulent)	Rickettsial Strains: E (avirulent)	Specific Loss[c]
Unfused L-929 Cells	42 ± 7[a] (100)[b]	38 ± 4 (100)	-
Residual Single Cells	42 ± 6 (100)	40 ± 7 (105)	-
L-L Heterokaryons	32 ± 4 (85 ± 11)	33 ± 4 (89 ± 2)	0.7
L-R Heterokaryons	23 ± 3 (60 ± 11)	10 ± 2 (28 ± 6)	1.8

a) Mean and standard error. Parameters were determined 24 h post-fusion, 45 h post-infection.

b) The numbers in parentheses are normalized to the unfused L-929 cells in each experiment.

c) Specific Loss = % loss with E strain ÷ % loss with Breinl strain.

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